Effects of long-term estradiol treatment on the contractile response to muscarine and muscarinic receptor subtypes in the bladder of aged female rats

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ABSTRACT
In this study, we tried to elucidate the effect of estrogen treatment on the detrusor contractile response to muscarine and muscarine receptor subtypes of the bladder in 13-month-old female Wi-star rats. The rats were divided into two groups, controls and rats treated with estradiol for 12 weeks. After the treatment phase, we monitored micturition behavior in addition to performing cystometrograms after the administration of muscarine, and real-time polymerase chain reaction for mRNA expression of the muscarinic receptor subtypes in the detrusor muscle. Our data indicated that there was a significant increase in the maximum micturition volume in the estradiol-treated rats. The urodynamic results indicated significant changes in the maximum detrusor pressure following the administration of muscarine in the estradiol-treated rats, in contrast to the controls for which no significant changes were observed. Furthermore, M3 receptor mRNAs in the detrusor muscle were significantly decreased in the estradiol-treated rats as compared to the control rats, while there were no differences noted for the M2 receptor mRNAs. Our data demonstrates that long-term estradiol treatment might be capable of increasing the potential detrusor contractility, and thus, estradiol might be a therapeutic agent that can be used to target the M3 receptors during the treatment of detrusor instability.

The most common type of urinary incontinence is stress urinary incontinence, which is defined as “the involuntary leakage on effort or exertion, or on sneezing or coughing.” Its prevalence rate in women ranges from 6.4% to 35% (9, 10). Urge incontinence is defined as “involuntary leakage of urine that is associated with a sudden, strong desire to void”, whereas mixed incontinence is regarded as “a combination of both urge and stress incontinence”. Studies have shown that both mixed and urge incontinence increases steadily with age. Thus, urinary incontinence is a common disease in elderly women with physical and/or psychosocial implications.

Clinical observations suggest that menopause contributes significantly to the onset of female urinary incontinence. One of the conservative therapies used to treat mixed urinary incontinence in women is estrogen replacement therapy (ERT). While alterations in female sex hormones appear to play a major role in mediating bladder dysfunction, the use of ERT in the management of post-menopausal urinary incontinence still remains controversial. A meta-analysis study reported there was a significant ERT effect on the subjective improvement of stress incontinence (6). However, in contrast to this, a more recent report has indicated that ERT worsened the characteristics of incontinence among symptomatic women (8).

Previous investigations have shown that ovariectomy decreased the detrusor muscle response to cholinergic agents in vitro, and that ERT increased...
the vesical pressure during voiding and bladder capacity in vivo (7, 12, 14, 19). The urinary bladder is very rich in muscarinic receptors of the M₂ and M₃ subtypes and they play a role in the contraction of the detrusor muscle (2). In a report by Shapiro, it was shown that ERT decreases the detrusor muscarinic receptor in ovariectomized rats (17). Thus, muscarinic receptor densities after ERT may possibly be linked to changes in the sensitivity of the bladder detrusor muscle. To our knowledge, there have been no reports on changes of the M₂ and M₃ subtypes due to ERT.

The purpose of this study was to determine the effect of estradiol on the urodynamic response to the administration of muscarine in vivo and to observe changes of the M₂ and M₃ subtypes in aged female rats.

MATERIALS AND METHODS

Animal model. For this study, 13-month-old female Wistar rats were maintained under standard laboratory conditions (12 h light/12 h dark, food and water ad libitum). The animals were randomly divided into two groups of 6 animals each. The estradiol-treated animals underwent daily subcutaneous injections of β-estradiol (20 μg/kg; Wako, Osaka, Japan) dissolved in sesame oil (0.2 mL/body; Sigma, St. Louis, MO, USA) for 12 weeks. Control rats only received subcutaneous sesame oil replacement (0.2 mL/day) for 12 weeks.

Monitoring micturition behavior. At 12 weeks after the estradiol treatment, all of the rats were put into a metabolic cage containing a urine collection funnel that was placed over an electronic balance (HF200; A.N.D., Tokyo, Japan) with the micturition behavior measured as has been described previously (18). Briefly, 250 mL plastic beakers were placed on balance pans. To monitor the cumulative weight of the collected urine, the balances were connected to a personal computer (Macintosh PowerBook) via a bridge amplifier (ML112; ADInstruments Pty. Ltd.) and a multiport controller (Maclab/400; ADInstruments Pty. Ltd.).

To establish a reliable baseline, we allowed at least 30 min after the surgery before the measurements were taken. The parameters measured included intercontraction interval between two voiding cycles (ICI), maximum detrusor pressure (Pdet, max), and the post-contraction resting pressure (RP). A 15 min cystometrogram recording was made for the purpose of obtaining a control phase, followed by stepped dosages of muscarine (3, 6, 10 μg/body; Sigma) that were injected via the femoral vein cannula.

Serum estradiol measurement and tissue sampling procedures. After the cystometrogram, 2.5 mL of blood was taken from the heart and placed in a cyclone separator and used to measure the plasma estradiol concentration. Plasma estradiol concentrations were assessed using a radioimmunoassay kit from SRL (Tokyo).

The urinary bladder was then rapidly dissected and weighed. The mid portion of the bladder body that did not contain urothelium was taken out and used for the real-time polymerase chain reaction (RT-PCR) procedure.

RT-PCR (quantification of muscarinic M₂ and M₃ receptor messenger RNAs). Muscarinic M₂ and M₃ receptor messenger RNAs (mRNAs) in the experimental bladder dome were measured using a RT-PCR method. The RNA was purified using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. A reverse transcriptase (RT) mixture (28 μL) containing 2 μg of total RNA was made and incubated at 37°C for 60 min according to a previously reported method (16). Five microliters of the mixture was used for RT-PCR, which was carried out using a LightCycler thermal cycler system with a LightCycler-FastStart DNA Master Hybridization Probe (Roche Diagnos-
Monitoring micturition behavior
The micturition behavior data for the experimental animals are shown in Table 3. There was no difference in urine production, average micturition volume and micturition frequency between the two groups. However, there was a significant increase in the maximum micturition volume in the estradiol-treated group as compared to the control group.

Cystometrogram
The ICIs in both groups were markedly shorter following the administration of muscarine (Fig. 1). In contrast to the control group, for which there were no significant changes, the Pdet,max was significantly increased by the intravenous administration of muscarine in the estradiol-treated group. There was also a tendency toward a dose-dependent increased RP in the estradiol-treated group, although the differences were not statistically significant.

Measurements of muscarinic M₂ and M₃ receptor mRNAs in the rat bladder dome
Table 4 shows the expression of muscarinic M₂ and M₃ receptor mRNAs in the rat bladder dome. Although there were no significant changes in the expression of M₂ mRNAs after the estradiol treatment, there was a significant decrease in the expression level of the M₃ mRNAs.

Table 1  Oligonucleotide primers and probes used for amplification of M₂ and M₃ muscarinic receptors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position</th>
<th>Oligonucleotide sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>M₂</td>
<td>(Primer) forward (1390–1409)</td>
<td>5’-CCACCTCCAGAGATGACACT-3’</td>
</tr>
<tr>
<td>M₂</td>
<td>(Primer) reverse (1519–1537)</td>
<td>5’-GCTCAACCGTCTC-GCTTTT-3’</td>
</tr>
<tr>
<td>M₃</td>
<td>(Primer) forward (1227–1245)</td>
<td>5’-GGACTGTGGATGTGAGAG-3’</td>
</tr>
<tr>
<td>M₃</td>
<td>(Primer) reverse (1358–1375)</td>
<td>5’-CGAGAGTTGAGTCAGA-3’</td>
</tr>
<tr>
<td>M₃</td>
<td>(Probe) forward (1433–1460)</td>
<td>5’-XACACATCACACTTTTTGGCCCTTGAGACT-3’</td>
</tr>
<tr>
<td>M₃</td>
<td>(Probe) reverse (1462–1494)</td>
<td>5’-XACACATCACACTTTTTGGCCCTTGAGACT-3’</td>
</tr>
<tr>
<td>M₂</td>
<td>(Probe) forward (1267–1284)</td>
<td>5’-XGT-CAGAAGGATTCACCAAGCTCCATCTC-3’</td>
</tr>
</tbody>
</table>

Table 2  General features of the experimental rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body [weight] (g)</td>
<td>320.0 ± 6.6</td>
<td>277.8 ± 31.7</td>
</tr>
<tr>
<td>Serum estradiol (pg/mL)</td>
<td>53.0 ± 8.6</td>
<td>164.0 ± 32.7*</td>
</tr>
<tr>
<td>Bladder [weight] (g)</td>
<td>0.108 ± 0.007</td>
<td>0.126 ± 0.005*</td>
</tr>
</tbody>
</table>

There were 6 rats per group. Each value represents the mean ± SEM. * Significantly different from the control group. ET: estradiol-treated rats

Table 3  Comparison of micturition behavior in control and estradiol-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine production (mL/day)</td>
<td>18.3 ± 2.2</td>
<td>20.4 ± 2.6</td>
</tr>
<tr>
<td>Micturition frequency (per day)</td>
<td>20.2 ± 3.1</td>
<td>15.6 ± 1.7</td>
</tr>
<tr>
<td>Average micturition volume (mL)</td>
<td>0.9 ± 0.1</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Maximal micturition volume (mL)</td>
<td>2.0 ± 0.1</td>
<td>3.1 ± 0.4*</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM. * Significantly different from control. ET: estradiol-treated rats

Statistical analysis. All results are expressed as the mean ± SEM and compared using an unpaired Student’s t test with p < 0.05 accepted as being statistically significant. Analyses were performed with the Statview statistical package (SAS Institute, Cary, NC, USA)

RESULTS

General features of the experimental animals
The data obtained regarding the general features, bladder weight and serum estradiol levels are shown in Table 2. While the estradiol-treated group showed no body weight gain, there was a significant increase in both bladder weight and serum estradiol concentration as compared to the control group.

Statistical analysis. All results are expressed as the mean ± SEM and compared using an unpaired Student’s t test with p < 0.05 accepted as being statistically significant. Analyses were performed with the Statview statistical package (SAS Institute, Cary, NC, USA)

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studies have demonstrated that ovariectomy induces a decreased muscle component of the bladder, whereas with ERT there is an increased muscle component (7, 13, 19). In the current study, the significant increase of the bladder weight we observed in the estradiol-treated group might indicate functional hypertrophy, similar to that reported in the other studies. While our urodynamic study failed to reveal any significant differences in the Pdet,max between the estradiol-treated rats and the control rats, we did observe a significant increase in the Pdet,max following the administration of muscarine in the estradiol-treated rats.

Functional studies in vitro have demonstrated an effect by ERT on the response of the detrusor when exposed to muscarinic agents (3, 10, 13, 14, 19). Longhurst et al. reported that ovariectomies significantly decreased the contractile responsiveness of the bladder body strips to nerve stimulation, ATP, carbachol, and KCl in young adult rats (14). In addition, ERT significantly improved the contractile responsiveness to these stimulations. These in vitro findings support our urodynamic results. We believe that the estradiol treatment increases detrusor contractility in aged female rats.

The effect of estrogen on the detrusor muscarinic receptor remains unclear. A previous report has shown that estradiol treatment for only 4 days increased the muscarinic receptor density in the rabbit...
Effects of estrogen on muscarinic receptor subtypes in the bladder

bladder body (11). However, there are recent reports that indicate there is a down-regulation of the muscarinic receptor density of the bladder body after estriadiol treatment in female rabbits in studies using radioligand receptor binding (3, 17). When using Furchgott’s double-reciprocal method, Liang et al. reported results that suggested that estrogen depletion might not contribute to a change in the affinity of the detrusor muscarinic receptor in the rat (12). We speculate that these discrepant results might be related to the different intervals of estradiol treatment and/or different methods that were used to measure the detrusor muscarinic receptor.

There have been no reports that indicate that long-term estradiol treatment alters the muscarinic receptor subtype in the detrusor muscle. There is a heterogeneous population of muscarinic receptor subtypes in many tissues, including the urinary bladder. A predominance of the M2 receptor subtype along with a smaller population of M1 receptors has been observed in the urinary bladder smooth muscle (15). Although both receptors influence contraction of the detrusor muscle, the M1 receptor appears to be the most functionally important, as it mediates direct contraction of the detrusor muscle (4). While our studies indicate that estradiol treatment seems to decrease the expression level of the M1 mRNAs, additional investigations are needed to further explain these results.

A previous study monitoring micturition characteristics found that the mean and maximal micturition volumes significantly increased following estradiol treatment in ovariectomized rats (14). Our data also showed that estradiol significantly increased the maximal micturition volume. We speculate that a decrease of the M1 receptor density may be responsible for inducing a reduction of the detrusor tone that leads to an increase in the bladder capacity.

In conclusion, our results demonstrated that the down-regulation of the M1 receptor following long-term treatment with estradiol might possibly improve the instability of the detrusor muscle and increase the detrusor contractility by muscarinic stimulation. Further studies are needed to delineate the specific mechanism by which these effects occur. In addition, our findings indicate that estradiol treatment may have therapeutic benefits for women with urge incontinence.

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REFERENCES


